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SHORT COMMUNICATION

INFLUENCE OF EXTRACTION PROCEDURE FROM EDIBLE MUSHROOM SPECIES *BOLETUS BADIUS* ON ZINC QUANTITY RELEASED INTO SIMULATED GASTRIC FLUID

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Research into the bioavailability and release of food components could play a significant role in health prophylaxis. For this reason, methods usually used for dissolution testing of pharmaceuticals should also be applied to food.

One of the oldest methods used for *in vitro* dissolution testing is the application of paddle apparatus (1). A second, also commonly applied method is flow-through analysis, where material for extraction is set into a thermostatic flow through cell, through which simulated gastric fluid or simulated intestinal fluid is pumped (1). The fluid movement is induced by various types of pumps – usually piston or peristaltic. Currently, it is thought that the flow through design (allowing better control over dissolution conditions, e.g., changing media) gives better results, which are easier to compare with the dissolution values from *in vivo* testing. In particular, modified semi-open flow-through designs seem to be the most promising in obtaining *in vitro* / *in vivo* correlations (2-4).

Furthermore, the assessment of substances in extracts obtained from dissolution experiments is also important. When metals are determined, it is necessary to go through a mineralization procedure – which is one of the most essential elements of quantitative analysis. Choosing the right conditions of mineralization is crucial; this depends on both the matrix used for the experiment and the determined component. The breaking and oxidation of organic bonds in the sample without any loss of analyte is crucial in this process. There are two types of mineralization: wet and dry. Wet mineralization is more common, and this consists of initiating sample reaction with one or more mineral acids and oxidative compounds by means of transferring energy to the sample. Given the losses of analyte that can occur during sample preparation, high-pressure, closed mineralization systems are currently preferred. Transfer of energy to the sample mixed with mineralizing agents can be done with UV light irradiation (emanating from low- or high-pressure mercury-vapor lamps.) This method usually gives the best results for liquid samples without large quantities of organic matter, for example: tap water, waste water (5, 6). Currently, microwave assisted digestion is most frequently applied and it is considered to be the best method of mineralization. This process is performed in concentrated mineral acids or their mixtures with oxidation agents (hydrogen peroxide). Large increments of temperature and pressure due to microwave energy increase the efficiency of this kind of mineralization. On the other hand, closed system and chemically passive Teflon vessels provide low losses of analyte and low background levels (7). There have been experiments using mixed types of mineralization where multiple energy sources are applied to both open and closed systems (8, 9).

Fruiting bodies of the edible mushrooms are rich in different physiologically active compounds

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(10, 11). One of them *Boletus badius* Pers. (bay bolette) grows in autumn in coniferous and mixed forests of Europe and North America. *B. badius* is a popular edible species due to its fragrance which is similar to *B. edulis* (King bolette). *B. badius* was also the first mushroom species analyzed for phenol acids and cinnamic acid quantity (12-16). In addition, it is a species where most of these metabolites can be found. Phenol compounds have a considerable antioxidative action, which explains the high total antioxidative activity of *B. badius* extracts described by Elmastas in 2007 (99.2% of inhibition in oxidation tests for 100 µg/mL methanol extract of dried fruiting bodies) (17). *B. badius* contains multiple neurotransmitter precursors and neurotransmitters: L-tryptophan (0.68 mg/100 g d.w. - dry weight), serotonin (0.52) and tryptamine (0.47) (18-21). *B. badius* fruiting bodies are also particularly abundant in free amino acids: tryptophan, cysteine, methionine, lysine, aspartate and glutamate acids. In lipid fractions, fatty acids are found, such as: linoleic (approx. 70%), palmitic (20%), oleic, lauric, miristic and arachidic (10). Mushroom fruiting bodies have an unusual trait among other living organisms, i.e., an ability to absorb and accumulate metals from the environment. Due to the presence of metallothioneins, *B. badius*, similarly to other mushroom species, has an excellent ability to accumulate elements such as zinc (22, 23). The highest ability of essential element accumulation (also those with antioxidant activity) is shown by *B. badius* fruiting bodies and spores. The content of these elements in sample material from these fruiting bodies was previously assayed with atomic absorption spectrometry and ranges between 137.6-219.6 µg/g d.w. for zinc, 34.9-59.9 µg/g d.w. for copper, 163.0-457.1 µg/g d.w. for iron and 795.5-1000.0 µg/g d.w. for magnesium (24).

The typical mechanism of accumulation in mushrooms occurs in the following manner: elements absorbed from the environment are bound with metallothioneins. There are numerous publications about research on metal quantity in mushroom fruiting bodies, but (as in the case of food) there are no data about their release in the human organism (24, 25). Unlike food, dissolution testing of pharmaceuticals and release experiments are routine (1). Given these facts, the main goal of this experiment was to adapt drug dissolution testing methods to the extraction of mushroom material in simulated gas-

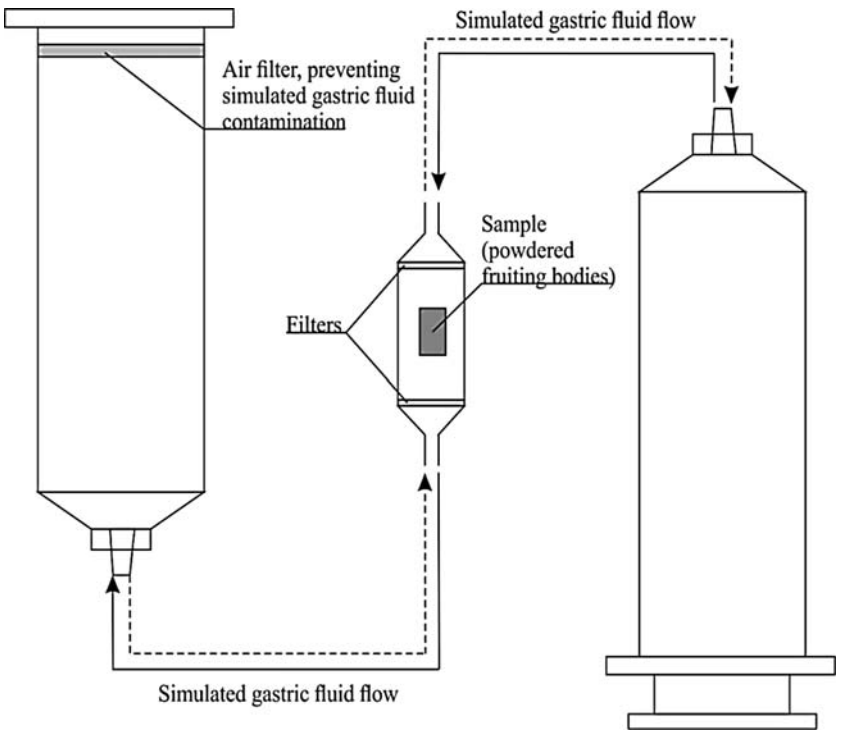

Figure 1. Single line of G-flow-2015 apparatus, only one of six parallel channels for dissolution testing was shown

tric fluid. Secondly, a model simulating conditions in the human digestive tract was created and the procedure for obtaining extracts was optimized.

EXPERIMENTAL

Reagents and standards

In the experiment, concentrated mineral acids: HCl, HNO_3 and dry salts; KCl, KNO_3 and 30% H_2O_2 solution were used and all were Suprapur® grade (Merck, Darmstad, Germany). Standard solutions of zinc 1 µg/mL and 10 µg/mL were prepared by appropriate dilution of 1000 mg/L stock standard solution (Okręgowy Urząd Miar, Łódź, Poland). For simulated gastric fluid, pepsin (BTL sp. z o.o., Łódź, Poland) was used. Quadruple distilled water with a conductivity lower than 1 $\mu S \times cm^{-1}$ was prepared in an S2–97A2 quartz distilling unit (Chemland, Stargard Szczeciński, Poland). Simulated gastric fluid was prepared according to Polish Pharmacopeia XI. Two grams of sodium chloride and 3.2 g of pepsin were dissolved in a small amount of quadruple distilled water, next, 80 mL of 1 mol/L hydrochloric acid was added to reach the correct pH (1–3) and the total volume was made up with quadruple distilled water to 1 L (25).

Research material

Boletus badius fruiting bodies collected in 2013–2014 in forests of southern Poland were used in experiments. The fruiting bodies were classified according to Knudsen and Vesterholt (26) by Muszyńska from the Department of Pharmaceutical Botany, Jagiellonian University Medical College. Fresh mushrooms were lyophilized (Freezone 4.5, Labconco) at -40°C.

Preparation of *Boletus badius* fruiting body extracts for use in simulated gastric fluid

Lyophilized fruiting bodies were ground in an agate mortar and accurately weighed into approx. 500 mg portions. The mushrooms were divided into two groups.

Extraction in a Gastroel-2014 apparatus

The mushrooms were incubated in simulated gastric fluid in a Gastroel-2014 – an apparatus that simulates peristaltic movement – created in the Department of Inorganic and Analytical Chemistry, Faculty of Pharmacy, Jagiellonian University Medical College. Five hundred milligrams of mushroom material were put into an Erlenmeyer flask, in which 30 mL of simulated gastric fluid was added and incubated at 37°C for 30 min. The solutions were filtered through MCE 0.22 µm ϕ 33 mm syringe filters.

Extraction in a G-flow-2015 apparatus

A second group of weighed portions was placed inside a G-flow-2015 apparatus, also created in the Department of Inorganic and Analytical Chemistry, Faculty of Pharmacy, Jagiellonian University Medical College. The apparatus consisted of thermostatic cells made from high density polyethylene (HDPE) and a syringe pump. Simulated gastric fluid was pumped through the cells to an expansion vessel by a Computer Numeric Controlled (CNC) six-way syringe pump. A simplified technological drawing of a single channel of the G-flow-2015 apparatus is shown in Figure 1.

Gastric fluid sample mineralization

Solutions obtained from dissolution apparatuses were divided into three parts. The first was directly mineralized; the second was filtered through an MCE 0.22 µm, ϕ 33 mm syringe filter; the last one was filtered through glass fiber paper. Solutions after filtering were mineralized by different methods.

UV light assisted digestion

Samples were closed in quartz vessels in a Mineral R-8 UV mineralization unit (Mineral, Poland) with an addition of concentrated nitric acid(V) or its mixture with hydrogen peroxide (10 : 1 diluted sample: nitric acid or 10 : 1 : 0.1 diluted sample: nitric acid : hydrogen peroxide). The total time of the samples mineralization was 48 h in 8 h cycles. After each 8 h, the lamp was switched off for cooling used apparatus.
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Microwave assisted digestion

Samples were closed in a Teflon vessel in a Magnum II microwave mineralization unit (Ertec, Poland). The mixtures of mineralizing agents were the same as for UV mineralization. Microwave mineralization was done in three stages: in the first, magnetron power was set to 60% for 10 min; the second took 15 min with 80% power; in the third one (15 min), power was set to 100%. The results from each type of mineralization are shown in Table 1.

Samples that were mineralized in UV and microwaves were quantitatively transferred to quartz evaporation vessels. Then, the fluid from the mineralizer was evaporated to almost dry residue and quantitatively transferred to 10 mL volumetric flasks and made up with quadruple distilled water.

Dry ashing in a muffle furnace

An FCF 7S muffle furnace with a standard temperature controller (Czylok, Poland) was used

Table 1. The quantity of zinc ions in samples subdued to various mineralisation techniques (G flow 2015). The results were shown as µg/g d.w. (dry weight taken for dissolution testing).

Mineralization factor	Microwave assisted mineralization	UV light assisted mineralization	Muffle furnace
µg/g d.w.			
HNO_3	77.9 ± 7.5	16.6 ± 1.3*	
HNO_3 and H_2O_2	63.5 ± 2.9	14.0 ± 0.9*	
Temp = 400°C			42.6 ± 0.9
Recovery	95.7%	89.4%	72.2%

n = 9, the mean ± SD, Statistica 10 (StatSoft, Poland), *diluted samples for UV irradiation.

for dry ashing. The samples were evaporated to dry residue and placed in a muffle furnace for 8 h at 400°C. The ash was dissolved in nitric(V) acid and treated in the same manner as extracts from UV light and microwave mineralization.

Voltammetric measurements

Mineralized samples were analyzed with differential pulse anodic stripping voltammetry (DP ASV). The measurements were made on an M161 multifunctional electrochemical analyzer with an M164 electrode stand (mtm-anko, Kraków, Poland). A standard three electrode setup was used, a controlled growth mercury drop electrode (CGMDE) as a working electrode (surface area of the mercury drop was 1.2 mm^2), a double junction silver/silver chloride electrode as a reference electrode (inner junction 3 mol/L KCl, outer 2 mol/L KNO_3), and platinum wire was used as an auxiliary electrode. The volume of the voltammetric cell was 20 mL; all measurements were done in 0.2 mol/L KNO_3 supporting electrolyte. Measurements were made between -1300 to -900 mV, accumulation potential was set to -1400 mV and the accumulation time to 20 s, the step potential was 4 mV at a 20 mV amplitude. Prior to analysis, the solution in the cell was deaerated for 3 min with a constant argon flow. After registering the background signal, 20–500 µL of sample were added to the voltammetric cell with 5 mL supporting electrolyte and voltammograms were registered. Next, three additions of zinc standard solution were added to the cell. With an increase in current, the concentration of zinc in the sample was calculated. Results were shown as µg of zinc released from 1 g of d.w. of lyophilized fruiting bodies.

Validation of voltammetric method and optimization of digestion

The procedure for zinc determination in extracts from dissolution testing after 30 min of extraction in a G-flow-2015 apparatus was validated according to ICH Q2(R1) criteria. Precision, selectivity linearity and recovery were calculated. Precision was estimated from nine measurements (n = 9) of the same sample and calculated as a relative standard deviation of these (RSD). Limit of detection and limit of quantification were calculated according to the formulae: LOD = $3 \cdot s \cdot a^{-1}$, and LOQ = $10 \cdot s \cdot a^{-1}$, where s – standard estimation error of linear regression slope. The values of LOD and LOQ were as follows: LOD = 0.36 µg/L and LOQ = 1.2 µg/L. The selectivity of the method was estimated by measurement of zinc ions in a sample spiked with standard solutions of copper(II) cadmium(II) and lead(II). Copper(II) cadmium(II) and lead(II) were selected, because these metals (especially copper) can form intermetallic compounds with zinc in drops of mercury, what leads to a substantial reduction of the accuracy and precision of the measurement. It was shown that an addition of these ions as high as 100 µg/mL did not interfere with the determination of zinc in trace amounts. The accuracy of the method was estimated based on zinc recovery from the sample processed by different methods of mineralization. A sample with known concentration of zinc ions was spiked with Zn(II) standard solution 100 µg/mL in increasing amounts, corresponding to 50, 100 and 150% of the initial amount. For each addition three measurements were made. UV mineralization gave a mean recovery of 89.4%. The lowest recovery was recorded for mineralization in a muffle furnace (72.2%). The highest recovery was observed for microwave assisted mineralization, where the mean value of recovery was 95.7%. The calibration curve was linear from 1 to 100 µg/L, the regression line was described as $y(\mu A) = -0.104C_{Zn}$ (µg/L) + 0.016. The calculated Pearson's correlation coefficient was r = 0.996.

Data analysis

All statistical calculations were made in Statistica 10.0 (StatSoft). To determine the signifi-

cance of differences observed for various mineralization techniques and sample preparation ways, Fisher-Snedecor and t tests were used at $\alpha = 0.05$.

RESULTS AND DISCUSSION

The results obtained during the measurements point to the fact that different mineralization procedures strongly influence the concentrations of zinc in simulated gastric fluid extracts from mushroom material. Between the results from the muffle furnace, UV light mineralization unit and the microwave assisted mineralization there are statistically significant differences. During the experiment, zinc was assayed in extracts obtained from 500 mg of *B. badius* in simulated gastric fluid. The results were recalculated and given as the amount of zinc extracted from a single gram of dry weight of lyophilized fruiting bodies ± standard deviation.

The oldest method of mineralization – dry ashing – gives the lowest recovery of zinc. This is probably caused both by an additional process of evaporation of extract and by a lengthy process of heating in a muffle furnace (theoretically, there is a possibility of contamination of the sample with zinc, but considering the recovery below 100%, it was not the case). The temperature used for dry ashing (400^{o}C), according to literature data, should not produce significant losses of zinc, but nevertheless a recovery of 70% disqualifies dry ashing from application with the extracts examined in this work.

Mineralization with UV light, frequently used for liquid samples, gave much better results. The recovery for this type of mineralization was almost 90% which cannot be considered an optimal value, but it is possible for trace analysis. When it comes to this method, the choice of substances added to the sample is also important. Mineralization for 48 h only with concentrated nitric(V) acid and diluted sample allowed the determination of 16.6 µg/g d.w. Application of the same mineralization period, but with a mixture of nitric acid and hydrogen peroxide gave 14.0 µg/g d.w., which is a statistically significant difference between these two mineralization factors. It can be stated that adding a strong oxidation agent significantly changes the results obtained during analysis.

Mineralization with microwave energy is effected in a significantly shorter time and the decomposition of organic matter is complete. Mineralization with 2 mL of concentrated nitric(V) acid gave 77.9 µg/g d.w., mixing the nitric acid with hydrogen peroxide gave 63.5 µg/g d.w., the mixture of nitric(V) and hydrochloric acid yielded 69.2 µg/g d.w. No statistically significant differences were calculated for this kind of mineralization with various mineralization reagents. This can be explained by the overall high efficiency of this method. The results from all methods are collated in Table 1. In particular, the differences between different methods of mineralization are important. In samples of simulated gastric fluid with the same conditions the results from microwave assisted mineralization are significantly higher than for other methods. In samples mineralized by dry ashing the zinc concentration was 42.6 µg/g d.w.

The amount of zinc released (both in Gastroel-2014 and G-flow-2015) into simulated gastric fluid correlates with earlier results for total zinc amount in fruiting bodies of *B. badius* obtained by Muszyńska (after microwave assisted digestion) (18).

A separate subject of this research was the influence of various extraction methods on zinc levels released from the samples to simulated gastric juice. Extraction by incubation was compared to extraction in a flow through system. The extract obtained from G-flow-2015 was divided into three groups, for each of these a different method of preparation before mineralization was applied. Samples mineralized without any prior pre-treatment showed 85.3 µg/g. d.w. of zinc. Filtering the extracts through a syringe filter provoked a statistically significant increase in zinc amount (93.3 µg/g d.w.). No statistically insignificant changes were found for samples filtered through glass fiber paper (90.0 µg/g d.w.).

CONCLUSION

Summarizing, there are considerable differences between zinc concentrations found in simulated gastric fluid after various mineralization methods. The highest recovery was found for mineralization in a closed microwave heated vessel. In this case, the mixture composition of mineralization agents had only a minor influence. The application of filtering through a syringe filter (routinely used after dissolution testing of drugs – it is necessary for the UV spectrometric determination of the released drug) in the case of trace analysis causes a statistically significant increase in results. The amount of zinc ions released from 1 gram of *B. badius* fruiting bodies dry weight increases in a flow through system – which correlates with similar results for drugs used as a model in dissolution testing. The research showed that the fruiting bodies of *B. badius* can be a valuable source of zinc for a human organism.

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